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Coincident Plasmids and Antimicrobial Resistance in Marine Bacteria Isolated From Polluted and Unpolluted Atlantic Ocean Samples

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Sewage effluent and outfall confluence samples were collected at the Barceloneta Regional Treatment Plant in Barceloneta, Puerto Rico; outfall confluence samples at Ocean City, Md., were also collected. Samples from uncontaminated open ocean areas served as clean-water controls. Bacteria were enriched in marine broth 2216 amended with 1 μg of one of a set of chemicals selected for study per ml: nitrobenzene, dibutyl phthalate, *m*-cresol, *o*-cresol, 4-nitroaniline, bis(tributyltin) oxide, and quinone. MICs of the chemicals were determined individually for all isolates. Bacterial isolates were evaluated for resistance to nine different antibiotics and for the presence of plasmid DNA. Treated sewage was found to contain large numbers of bacteria simultaneously possessing antibiotic resistance, chemical resistance, and multiple bands of plasmid DNA. Bacteria resistant to penicillin, erythromycin, nalidixic acid, ampicillin, *m*-cresol, quinone, and bis(tributyltin) oxide were detected in nearly all samples, but only sewage outfall confluence samples yielded bacterial isolates that were resistant to streptomycin. Bacteria resistant to a combination of antibiotics, including kanamycin, chloramphenicol, gentamicin, and tetracycline, were isolated only from sewage effluent samples. It is concluded that bacterial isolates derived from toxic chemical wastes more frequently contain plasmid DNA and demonstrate antimicrobial resistance than do bacterial isolates from domestic sewage-impacted waters or from uncontaminated open ocean sites.

The incidence of antibiotic-resistant bacteria in aquatic environments has increased dramatically as a consequence of the widespread use of antibiotics by humans. This increase has resulted from a variety of factors, perhaps the most important of which is the selection for resistant strains and the ability of such strains to exchange plasmids encoding resistance. The high incidence of resistant bacteria has been documented for chronically polluted waters (5, 6, 32, 37). Such bacteria also occur in sewage (6, 16, 33, 38, 40, 42), rivers and marine waters (12, 13, 20, 29, 30, 32, 37, 39, 41; D. L. Glassman, Ph.D. thesis, University of Maryland, College Park, 1981), fresh water, and marine shellfish (6, 7).

It is known that bacteria can transfer resistance plasmids in situ to indigenous microflora (27). Interspecies and intergeneric transfer of R plasmids has also been shown to occur (8, 9, 27, 31, 34). For example, Patt et al. (36) and Sizemore and Colwell (37) reported evidence of plasmid transfer from *Escherichia coli* to marine bacteria and Guerry and Colwell (19) reported transfer from *E. coli* to estuarine bacteria. The potential for plasmid transfer is especially significant in view of the fact that many bacteria containing R plasmids, the possession of which is associated with antibiotic resistance, exhibit higher rates of survival in aquatic environments (14, 15, 24).

Several studies have focused on the association between plasmids and antibiotic (32, 39) or heavy-metal (35; L. H. Bopp, H. L. Ehrlich, D. A. Friello, and A. M. Chakrabarty, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, H(H)13, p. 138) resistance in enteric bacteria isolated from the environment. It is also well known that heavy-metal resistance in bacteria is associated with single or multiple drug resistance (1, 11; S. C. Tripp, T. Barkay, and B. H. Olson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, Q19, p. 207). However, very few studies of the spatial and temporal distribution of plasmids in natural environments have been reported.

Glassman (Ph.D. thesis) found that many estuarine bacterial isolates carried at least one plasmid. Similarly, Kobori et al. (26) concluded that plasmids are ubiquitous among psychrophilic and psychrotrophic bacterial isolates from McMurdo Sound, Antarctica. Hada and Sizemore (20), in a study of the marine vibrios of the Gulf of Mexico, found a higher incidence of plasmid-bearing strains at a polluted site than at an unpolluted site. In contrast, Burton et al. (4) studied bacteria isolated from river sediments and found no significant difference between polluted and unpolluted sites with respect to incidence of plasmid-bearing strains. Here, we report that natural waters exposed to toxic chemical wastes showed a higher coincidence of antibiotic-resistant bacteria and bacteria bearing multiple plasmids than did isolates from samples of domestic sewage-impacted or clean open ocean waters.

MATERIALS AND METHODS

Four different types of samples were collected and examined for antibiotic-resistant bacteria. Sewage effluent samples, designated SE, were collected directly from the Barceloneta Regional Treatment Plant at Barceloneta, Puerto Rico; effluent from this plant was not disinfected (chlorinated) at the time of sampling. Water surrounding the Barceloneta Regional Treatment Plant outfall diffuser was also sampled (samples designated SO) during this Puerto Rico cruise

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aboard the R/V *Mt. Mitchell* in February 1982 (18). In March 1983, additional seawater samples were collected from various sites around the outfall diffuser of the wastewater treatment plant in Ocean City, Md (samples designated OC), during a research cruise aboard the R/V *Cape Hatteras*. Sewage at Barceloneta Regional Treatment Plant is composed of 65% pharmaceutical and industrial waste; sewage at Ocean City (OC) is primarily domestic, with large volume fluctuations related to seasonal factors, notably summer tourist trade. Clean water (CW) samples from unpolluted, control sites were also collected from the same ship, at a location off Beaufort, N.C. (lat. 36°35′ N, long. 75°30′ W), in May 1982. Sterile bag samplers (General Oceanics Inc., Miami, Fla.) were used to obtain all SO, OC, and CW water samples; SE was collected from a sampling faucet into sterile glass bottles.

Isolation and maintenance of strains. Each water sample (1 ml) was added to seven separate 2-ml volumes of marine broth 2216 (Difco Laboratories, Detroit, Mich.), each supplemented with 1 μg of nitrobenzene, *o*-cresol, *m*-cresol, quinone, 4-nitroaniline, bis(tributyltin) oxide, or dibutyl phthalate per ml. Cultures were maintained in the enrichment medium until testing. The testing of the strains was done as follows. After overnight incubation at 27°C, cultures were streaked onto marine agar 2216 plates. Isolates were picked and transferred to eight differential media (Difco Laboratories): xylose-lysine-deoxycholate, thiosulfate-citrate-bile salt-sucrose, MacConkey, MacConkey plus trehalose, staphylococcus 110, Levine eosin-methylene blue, pseudomonas P, and pseudomonas F agars. After incubation for 24 h at 27°C, colonies were selected at random and streaked for purity, and the pure cultures were maintained both in semisolid media (yeast extract, 3.0; peptone, 10; NaCl, 10; and agar, 5 g/liter) and in 12% aqueous glycerol under liquid nitrogen.

MIC. The MIC was determined for each strain and for all chemicals, using a solid medium (yeast extract, 3; NaCl, 10; peptone, 10; and agar, 18 g/liter) containing the following concentrations of chemicals: *o*-cresol and *m*-cresol, 500, 250, and 125 μg/ml; bis(tributyltin) oxide, 24, 12, and 6 μg/ml; quinone, 75, 37.5, and 12.5 μg/ml; dibutyl phthalate, nitrobenzene, and 4-nitroaniline, 1,000 and 500 μg/ml. The chemicals used were among those recommended for priority consideration under the Toxic Substances Control Act, and the concentrations were within the range of the maximum expected concentration of these pollutants in aquatic environments.

Antibiotic resistance. Antibiotic resistance testing was performed with Mueller-Hinton agar plates (Oxoid Ltd., USA, Columbia, Md.) and Sensidiscs (BBL Microbiology Systems, Cockeysville, Md.), following standard methods (2). The antibiotics tested were ampicillin (10 μg), chloramphenicol (20 μg), erythromycin (15 μg), gentamicin (10 μg), kanamycin (10 μg), novobiocin (30 μg), penicillin (30 μg), streptomycin (10 μg), and tetracycline (30 μg). Isolates were considered sensitive by standards suggested by Bauer et al. (2).

Screening for plasmid DNA. Cultures were incubated overnight on Upper Bay yeast extract agar plates (1), and plasmid DNA was alkaline extracted by the method of Kado and Liu (23). Agarose (0.7%) gel electrophoresis was performed, using a water-cooled horizontal apparatus (HGE 1312; Savant Instruments, Hicksville, N.Y.). After 4 h, gels were submerged in ethidium bromide solution (1 μg/ml; Sigma Chemical Co., St. Louis, Mo.) for 25 min and then allowed to stand in distilled water overnight at 4°C. Gels were subsequently visualized with a 300-nm transilluminator (model 3-4400; Fotodyne, New Benton, Wis.) and photographed with Polaroid 665 film exposed through Wratten filters (no. VP29 and 2B). The molecular weights of the plasmids were determined by comparing the R_f values of the unknown plasmid DNA to a standard curve of molecular weight versus R_f obtained with *E. coli* strain V517 (28).

Computer analysis. With the exception of data concerning plasmid molecular weights and chemical enrichments, each trait was coded for present, absent, or unknown. There were four possible outcomes for each MIC test, and the MIC data were coded by using a scheme devised by Beers and Lockhart (3).

Plasmid molecular mass data were coded as megadaltons (MDa), and these data were not used in the original cluster analysis. Similarly, the enrichment chemical was coded as a single-digit number from 0 (control) to 7 and was inspected only after clusters had been determined, in an attempt to discover whether cluster members shared chemical affinities.

All clustering was done with the program TAXAN6, available on the University of Maryland Univac 1108 computer. The data were analyzed by using the Simple Matching coefficient, S(M), which includes both positive and negative matches, and the Jaccard coefficient, S(J), which excludes negative matches. Clusters were determined by single and unweighted average linkage.

Feature frequencies and chi-square tests for association were performed by using the Statistical Analysis System on the University of Maryland IBM 4381. A number of reformatting and plotting functions were then performed by awk scripts. Tables, histograms, and bar charts were produced in a similar manner.

RESULTS AND DISCUSSION

Feature frequencies by site for the 229 strains isolated and tested in this study. The frequency of positive characteristics for the entire data set (229 strains), as well as a breakdown by site, is given in Table 1. Eighteen characteristics were used for feature frequency and cluster analysis. All strains proved to be gram-negative, rod-shaped bacteria.

Of the 38 oxidase-negative strains, 36 were isolated from SE. This source also yielded the majority of erythromycin-, streptomycin-, and kanamycin-resistant strains, as well as the only strains isolated in the study that were resistant to gentamicin, chloramphenicol, or tetracycline. SE and SO strains were highly resistant to *m*-cresol and quinone, although >50% of all strains were resistant to concentrations of 250 to 500 μg of *m*-cresol per ml. Strains from all sites were resistant to lower concentrations of bis(tributyltin) oxide than to the other chemicals tested. Strains isolated from samples collected at the control sites (CW), in particular, were shown to be sensitive to this compound.

The chi-square test for homogeneity of proportion indicated a statistical difference ($P < 0.05$) between sites for all characteristics tested, with the exception of Gram reaction and catalase production. Pairwise chi-square tests were performed for each characteristic, to determine which sites were responsible for the differences observed. These results are also presented in Table 1. In these pairwise tests, the significance level was adjusted to create a Bonferroni multiple-comparison procedure (25). A Kruskal-Wallis test (21) was used to compare number of plasmids and molecular weights with a corresponding multiple-comparison procedure to determine which sites were different.

TABLE 1. Feature frequencies (percent) for complete data set and breakdown by site[a]

Trait	Data set (n = 229)	CW (n = 42)	OC (n = 60)	SO (n = 40)	SE (n = 87)
Basic tests					
Gram					
Negative	98.69	100.00[a]	100.00[a]	95.00[a]	98.85[a]
Positive	0.44	0.00	0.00	0.00	1.15
Oxidase	83.41	100.00[a]	96.67[a]	100.00	58.62[b]
Catalase	95.63	100.00[a]	96.67[a]	92.50[a]	94.25[a]
Glucose oxidation	72.05	52.38[a]	88.33[b]	87.50[b]	63.22[a]
Glucose fermentation	59.39	38.10[a]	78.33[b]	87.50[b]	43.68[a]
Plasmids	44.98	16.67[a]	13.33[a]	47.50[c]	79.31[d]
Antibiotic resistance					
Penicillin	60.26	23.81[a]	68.33[b]	55.00[b]	74.71[b]
Erythromycin	27.51	0.00[a]	3.33[a]	5.00[a]	67.82[b]
Ampicillin	40.81	14.29[a]	50.00[b]	37.50[a,b]	47.13[b]
Chloramphenicol	5.68	0.00[a,b]	0.00[a]	0.00[a,b]	14.94[b]
Streptomycin	16.16	0.00[a]	0.00[a]	15.00[a,b]	35.63[b]
Tetracycline	2.62	0.00[a]	0.00[a]	0.00[a]	6.90[a]
Kanamycin	9.61	0.00[a]	0.00[a]	2.50[a]	24.14[b]
Novobiocin	37.99	4.76[a]	53.33[b]	22.50[a]	50.58[b]
Gentamicin	3.93	0.00[a]	0.00[a]	0.00[a]	10.35[a]
MIC[b]					
m-Cresol					
L	1.31	0.00[a]	5.00[a]	0.00[b]	0.00[b]
M	9.61	7.14	20.00	2.50	6.90
H	60.26	78.57	58.33	60.00	52.87
X	19.65	2.38	1.67	30.00	35.63
Bis(tributyltin) oxide					
L	39.30	66.67[a]	31.67[b]	65.00[a]	19.54[c]
M	13.10	7.14	16.67	15.00	12.64
H	14.85	7.14	26.67	5.00	14.94
X	23.14	4.76	10.00	7.50	48.28
Quinone					
L	5.68	23.81[a]	5.00[b]	0.00[b]	0.00[c]
M	23.14	42.86	25.00	35.00	6.90
H	31.88	11.90	50.00	32.50	28.74
X	29.69	7.14	5.00	25.00	59.77

[a] Percentages with the same superscript are not significantly different, using a chi-square test with the Bonferroni multiple comparison method of adjustment of type I errors.

[b] L, Low (< = lowest concentration); M, medium (< = middle concentration); H, high (< = highest concentration); X, out of range (> highest concentration). Note: percentages add to less than 100% because MIC was not computed for several samples. Actual counts were used to compute statistics.

Strain clustering. The 229 strains examined formed a large group at the S (similarity) ≥31% level, using the Jaccard similarity coefficient and unweighted average linkage. Within this group, 10 clusters, or phena, were distinguished at varying levels of similarity above 65%. Twenty-one strains did not fall into any one of these phenetic groups. No cluster with less than five members was considered further. A shaded similarity matrix, showing all strains, is presented in Fig. 1A and a corresponding dendrogram showing all strains is presented in Fig. 1B. A comparison of positive characteristics among the clusters is shown in Table 2.

Clusters 1 and 10 were composed entirely of strains from SE. No other clusters contained strains from only one site. In addition, strains in cluster 1, which formed at the 68% level, were highly resistant to the chemicals quinone, bis(tributyltin) oxide, and m-cresol. All strains in the cluster possessed plasmid DNA.

Cluster 2 joined at S ≥ 75% and showed resistance to the highest concentration of all three toxic chemicals. Members of the cluster also showed very broad antibiotic resistance patterns, with 1 strain demonstrating resistance to eight of the nine antibiotics tested and 14 strains showing resistance to five or more. Only one-third of the strains possessed plasmid DNA.

Cluster 10 formed at 86%. All members of the cluster exhibited resistance to four or more antibiotics. Five of the nine gentamicin-resistant strains isolated during the study belonged to this cluster.

Cluster 3 joined at S ≥ 74% and was, in turn, joined by cluster 4 at 63%. All strains tested were resistant to novobiocin. Resistance to penicillin, ampicillin, and erythromycin was also observed. Three strains showed resistance to all four antibiotics.

Cluster 4 contained six strains isolated from SE, along with three strains from the SO samples. Antibiotic and toxic chemical resistances were lower in this cluster than in those previously described.

Cluster 5, which joined at S ≥ 73%, consisted primarily of strains from the CW control site. Only m-cresol resistance was high, with 72% of the cluster members showing a MIC 250 to 500 μg/ml. Furthermore, only two strains exhibited antibiotic resistance, both to novobiocin.

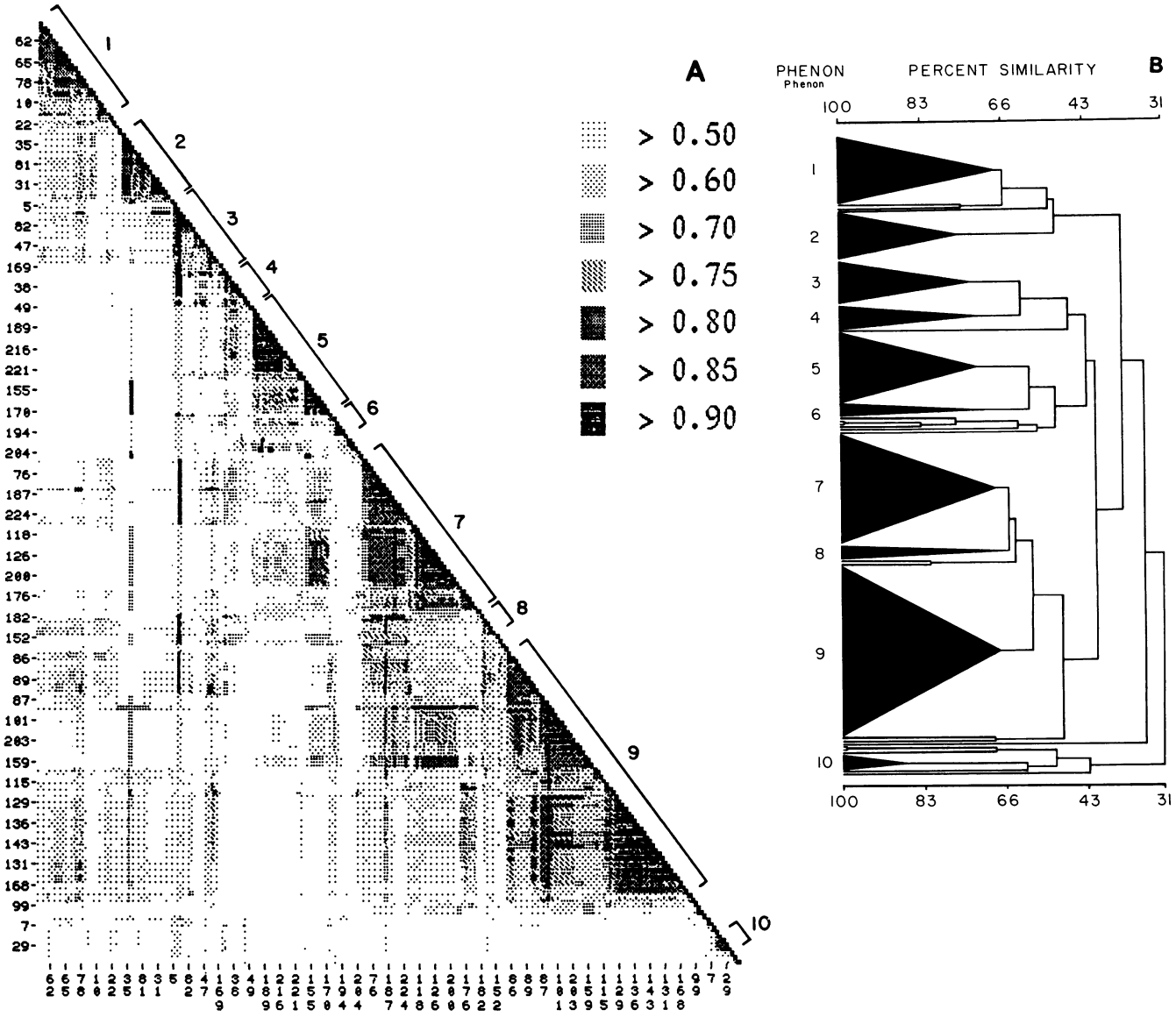

FIG. 1. Shaded similarity matrix prepared using the Jaccard coefficient and unweighted average linkage (A) and corresponding dendrogram (B).

Members of cluster 6 represented each of the four sites surveyed and exhibited similarity at ≥68%. No strong trends of toxic chemical or antibiotic resistance were observed within this group, although >80% of the members displayed a MIC of 250 to 500 μg of *m*-cresol per ml.

Cluster 7, which contained only five members, showed an internal similarity of ≥69%. One strain, from the SE site, possessed plasmid DNA. The other strains were from CW. All strains were resistant to penicillin and toxic chemical resistance for this group was low.

Cluster 8, with only six members, showed a 66% similarity and joined cluster 7 at 65%. All members possessed plasmid DNA and all but one of the strains tested were resistant to quinone at concentrations above 75 μg/ml.

The largest cluster, cluster 9, was composed of 61 strains, primarily from the OC and SO sites. Less than 20% of the strains contained plasmids. All exhibited some antibiotic resistance, with 26 strains showing resistance to penicillin, ampicillin, and novobiocin, while 22 strains were resistant only to penicillin and ampicillin. This cluster showed an overall similarity of 67%.

Antibiotic resistance, MIC, plasmid relationships. No strong correlation was found between number of plasmid bands, or average plasmid molecular weight, and antibiotic resistance pattern (Table 3). Of 18 strains showing the highest resistance overall, 10 had no detectable plasmid bands. It is possible that plasmids were present, but in low copy number, thereby escaping detection. It is also possible that very large plasmids (>100 megadaltons) went undetected, using the methods described. No particular size range of plasmid DNA could be correlated with any resistance combination.

Reasons other than plasmid-mediated resistance for increased antibiotic resistance of strains isolated from waters impacted by chemical wastes must be considered. Bacteria accomplish antibiotic resistance by different means. One mechanism is metabolism of the drug itself. Other possible mechanisms include decreased cell wall permeability and

TABLE 2. Characteristics of clusters

Feature	% of strains positive in cluster (no. of strains):									
	1 (25)	2 (17)	3 (16)	4 (9)	5 (25)	6 (5)	7 (39)	8 (6)	9 (61)	10 (5)
SE	100	94	63	67	8	20	13	17	5	100
SO	0	0	6	33	4	0	38	33	28	0
OC	0	0	19	0	32	0	21	33	59	0
CW	0	6	13	0	56	80	28	17	8	0
Oxidase	17	100	100	89	100	100	100	100	100	0
Catalase	92	100	100	100	100	100	100	100	100	100
Glucose oxidation	100	100	21	0	32	0	95	100	98	0
Glucose fermentation	100	0	13	0	0	0	100	100	98	0
Plasmids	100	24	100	100	0	20	42	100	18	100
Penicillin	96	100[a]	72	0	0	100	3	83	100	100
Erythromicin	21	100	64	38	0	0	0	33	3	100
Ampicillin	54	100	29	0	0	40	0	0	88	0
Chloramphenicol	0	50	0	0	0	0	0	0	0	17
Streptomycin	25	75	0	0	0	0	0	17	8	100
Tetracycline	4	19	0	0	0	0	0	0	0	17
Kanamycin	8	75	0	25	0	0	0	0	2	50
Novobiocin	67	82	100	0	8	0	16	0	50	0
Gentamicin	0	13	0	13	0	0	0	0	0	83
m-Cresol (μg/ml)										
< =125	0	0	0	0	0	0	0	0	4	0
< = 250	11	0	0	13	16	0	5	0	16	0
< = 500	46	0	69	88	72	60	82	80	70	83
> = 500	42	94	13	0	0	0	13	20	11	0
Bis(tributyltin) oxide (μg/ml)										
< = 6	0	6	13	75	72	40	73	60	41	50
< = 12	0	0	44	13	16	20	11	40	14	0
< = 24	0	0	9	0	0	0	14	0	32	33
> = 24	100	88	6	13	0	0	3	0	13	0
Quinone (μg/ml)										
< = 12.5	0	0	0	0	20	20	11	0	5	0
< = 37.5	0	0	13	25	48	20	43	20	27	0
< = 75.0	0	0	31	63	20	20	41	0	59	50
> = 75.0	100	94	38	13	0	0	5	80	9	33

[a] Based on $n = 14$.

alteration of the antibiotic receptor site; either of these is usually a result of a mutation(s) in the bacterial genome (10).

The relationship of plasmids to MIC was studied in a similar manner. Only 44% of the strains exhibiting tolerance to greater than the highest concentration of each chemical possessed plasmid DNA. However, all but one strain with this tolerance pattern were from SE. SE strains accounted for 65% of the strains resistant to the highest concentration of at least one chemical. SO strains made up an additional 20% of this group.

Comparison of antibiotic resistance patterns with MIC patterns showed a relationship between MIC levels and number of antibiotics to which the strain was resistant. In all but one case, strains which showed resistance to six or more antibiotics also exhibited MICs greater than the highest concentration for *m*-cresol, bis(tributyltin) oxide, and quinone. Strains with no antibiotic resistance tended to show low overall MICs.

Antibiotic resistance and presumptive genus. Of the 38 oxidase-negative strains in the data set, 24 were shown to be glucose fermentative. All were gram negative. Based on attributes described in *Bergey's Manual of Systematic Bacteriology* (22), these strains may be considered to be members of the family *Enterobacteriaceae*.

The 38 enteric strains were examined for plasmids, as well as for antibiotic resistance. Interestingly, comparison of number of strains with plasmids of $\log_{10}$ molecular weight for the enteric bacteria was similar to that of the complete data set. Plasmid bands with molecular sizes in the range 11.2 to 20 MDa were not detected within the enterics, and all but three strains were found to possess plasmid DNA. The number of plasmid bands per strain ranged from 1 to 11. These findings are significant especially in the finding of enteric bacteria with a high incidence of plasmids.

Furthermore, the antibiotic resistance patterns of these enteric strains were examined and compared with the oxidase-positive, glucose-oxidizing (*Pseudomonas* sp.) strains. Comparisons were also made to strains which were oxidase positive and facultative, i.e., oxidative as well as fermentative, and to strains which were neither oxidative nor fermentative (Fig. 2). Strains positive for oxidase and glucose fermentation (group III, Fig. 2) numbered 107 and comprised the largest group examined, demonstrating resistance to several antibiotics. Few strains in this large group demonstrated kanamycin resistance and none were resistant to chloramphenicol and tetracycline.

Plasmid relationships. Plasmid bands were found in bacteria isolated from samples collected from all sites, but the largest numbers of plasmid bands were found in strains isolated from SE samples (Fig. 3). Plasmid molecular sizes ranged from 1.4 to 151 MDa. The frequency of occurrence of

plasmid weight, indicated by $\log_{10}$ molecular weight and distributed by sites is presented in Fig. 4. A bimodal distribution is evident for SO, SE, and the entire set of strains. Plasmid bands in the molecular size range of 2.5 to 6.3 MDa were most common, while those between 12.6 and 20 MDa occurred much less frequently.

A statistically significant difference was found among sites ($P < 0.05$) for both number of plasmid bands and average molecular weight per strain. Paired comparison tests were performed to determine where differences were observed. As with chi-square tests on other features, a 0.008 significance level was set for the paired tests. No significant differences were found between CW and OC strains, for either average molecular weight per strain or average number of plasmids. SE strains were found to possess an average of 2.6 plasmid bands per strain, compared with 1.2 plasmid bands per strain for isolates from samples collected at the SO. Four or more plasmid bands were detected in 27% of the strains isolated from SE. The OC and CW isolates each revealed an average of one or less plasmid bands for every two strains.

Occurrence of antibiotic-resistance within species groups. Strains isolated in this study were separated into four groups on the basis of ability to oxidize or ferment glucose and oxidase reaction. Oxidase-negative fermenters were, for practical purposes of the study, identified as enteric bacteria. As indicated previously, oxidase-positive oxidizers were presumptively identified as *Pseudomonas* spp., while oxidase-positive, facultatively fermenting isolates were presumptively identified as *Vibrio* spp. (17, 22). The remaining strains in the data set did not oxidize or ferment glucose.

Relationship of MIC to site of sample collection. A strong correlation was observed between MIC and sample collection site. Because all samples were collected in the early spring of 1982 and 1983, seasonal variation has been discounted, based on bacteriological data accumulated for approximately 15 cruises since 1972 to the Puerto Rico Trench (17). SE isolates yielded results showing a strong difference from those of any other site, with only one exception: strains isolated from SE and SO exhibited similar resistance patterns for *m*-cresol.

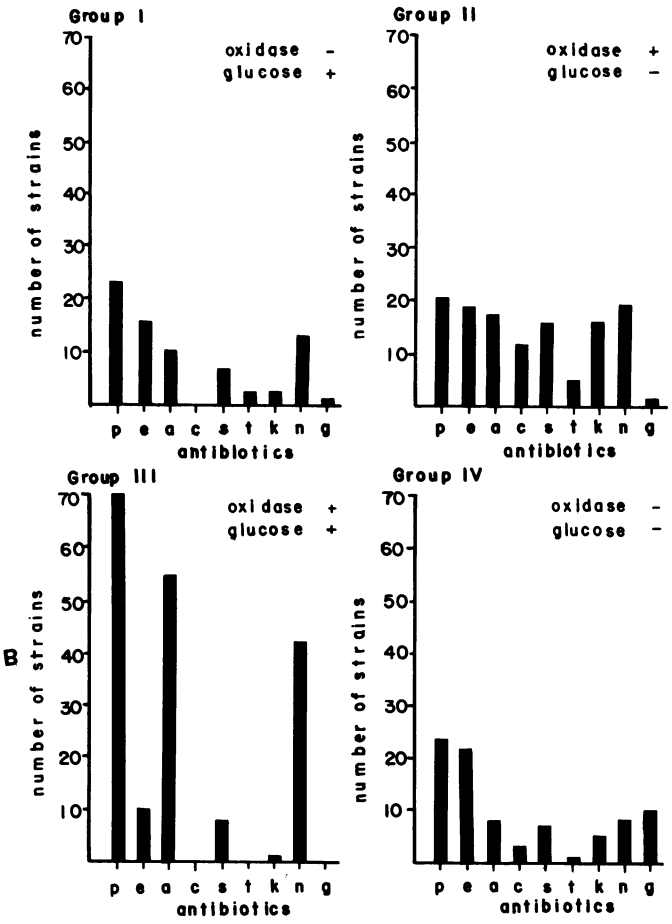

FIG. 2. Frequency of resistance to antibiotics for bacterial strains clustered in group I (oxidase negative, glucose fermentative), group II (oxidase positive, glucose negative), group III (oxidase positive, glucose positive), and group IV (oxidase negative, glucose negative). Antibiotic abbreviations: p, penicillin; e, erythromycin; a, ampicillin; c, chloramphenicol; s, streptomycin; t, tetracycline; k, kanamycin; n, novobiocin; g, gentamicin.

TABLE 3. Relationships between plasmids and antibiotic resistance for 229 strains

Antibiotic	No. of strains	No. with plasmids	Avg plasmids per strain	Avg mol size of plasmids (MDa)
Resistant strains:				
Penicillin	138	67	1.5	36.8
Ampicillin	92	38	1.1	39.2
Novobiocin	87	40	1.3	35.0
Erythromycin	63	48	2.6	31.7
Streptomycin	37	19	1.7	24.1
Kanamycin	22	12	2.2	10.8
Chloramphenicol	13	5	0.4	2.5
Gentamicin	9	8	3.7	36.0
Tetracycline	6	4	1.2	11.7
Sensitive strains:				
Penicillin	85	31	1.1	28.8
Erythromycin	160	50	0.9	36.8
Ampicillin	131	60	1.5	31.2
Chloramphenicol	210	93	1.4	36.0
Streptomycin	186	79	1.3	36.7
Tetracycline	217	94	1.4	35.2
Kanamycin	201	86	1.3	37.6
Novobiocin	136	58	1.4	33.8
Gentamicin	214	90	1.3	34.1

The highest MICs were recorded for strains isolated from SE samples. About 25% of the isolates from SE demonstrated a MIC greater than that of the highest concentration of each of the chemicals tested.

If the MICs for isolates from CW samples are taken as the reference, it can be seen that all bacteria, even those isolates from samples collected in uncontaminated ocean areas, showed some resistance to the toxic chemicals examined in this study. Resistance to dibutyl phthalate, nitrobenzene, and 4-nitroaniline among all strains was relatively high, i.e., $\geq$100 μg/ml. Higher concentrations of the compounds were not used.

It is concluded that pollution of ocean areas with toxic chemicals results in an increased tolerance of the bacterial populations to those chemicals. Whether these bacterial populations were allochthonous or autochthonous remains to be resolved. Nevertheless, one can conclude that selection for resistant strains will occur.

In summary, the data gathered in this study demonstrate a strong correlation among site of sample collection, resist-

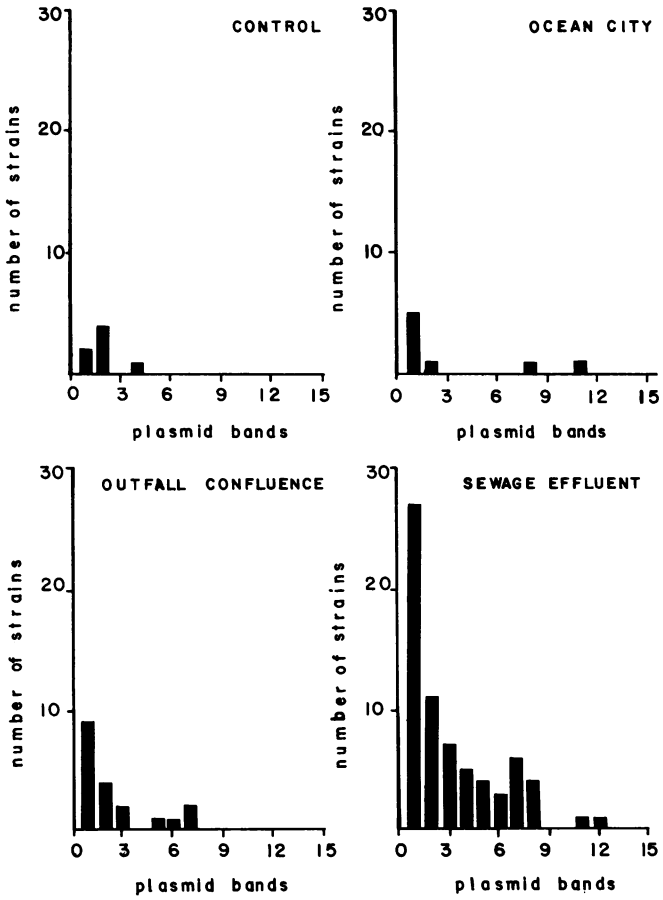

FIG. 3. Frequency of occurrence of the number of plasmids in individual bacterial strains isolated from water samples collected at: the CW (control) site, n = 42 strains, mean bands per strain = 0.33 (35 strains had no bands); OC site, n = 60 strains, mean bands per strain = 0.43 (52 strains had no bands); SO site, n = 40 strains, mean bands per strain = 1.2 (21 strains had no bands); and SE site, n = 87 strains, mean bands per strain = 2.6 (18 strains had no bands).

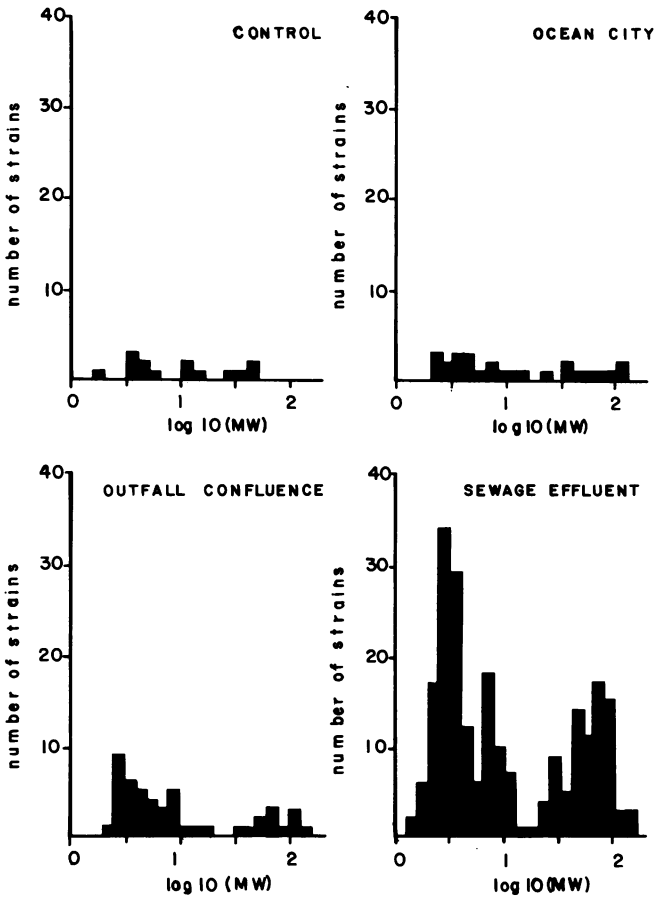

FIG. 4. Frequency of occurrence of plasmids in bacterial strains, $\log_{10}$ molecular weight (MW) isolated from water samples collected at the unpolluted, i.e., CW (control), site, OC site, SO site, and SE site.

ance to antibiotics and toxic chemicals, and incidence of plasmid DNA. For most traits examined in this study, isolates from SE comprised the largest group of chemical tolerant bacteria. Antibiotic resistance, both qualitative and quantitative, and MICs were significantly higher for isolates from SE than for isolates from the other sites investigated in this study.

A pairwise comparison of the sites showed a clear trend; i.e., as the amount and type of sewage contamination varied, so did the plasmid DNA and resistance pattern observed for the isolates examined in this study. Organisms isolated from SE collected at the Barceloneta treatment plant exhibited the highest resistance. Isolates from samples collected at the Barceloneta SO showed lower levels of resistance, approximating values for isolates from samples of domestic sewage-contaminated water collected off Ocean City (OC samples). As expected, strains isolated from CW were the most sensitive to the antibiotics and toxic chemicals examined in this study, and these isolates contained a smaller number of plasmids.

We previously concluded that the dumping of large quantities of pharmaceutical and chemical wastes into the ocean results in changes in bacterial community structure and species composition (17). This study represents an extension of our earlier work and allows us to make a further conclusion about ocean disposal of wastes. The increase observed in the number of plasmid bands detectable in strains isolated from samples of chemically contaminated waters is dramatic and merits attention by public health authorities as a possible source of multiply antibiotic-resistant and potentially pathogenic bacteria.

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